Genetic and Anatomical Analyses of the Jumping Slugs

Final Report

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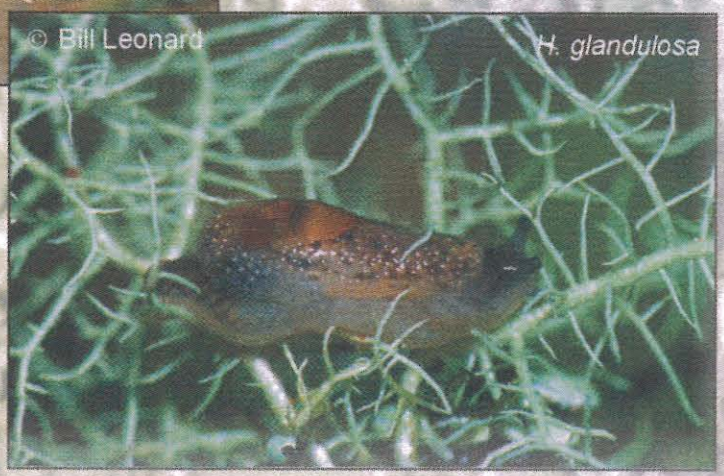

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1. Introduction

The genus *Hemphillia* (Gastropoda: Arionidae) is a slug taxon restricted to the Pacific Northwest. It is characterized by a visceral mass that is retained in a raised hump under the mantel, a shell plate that is not entirely enclosed (visible through a slit in the back of the mantle), a pneumostome in the right side of the mantle, a relatively narrow and little tapered tail, a caudal fossa near the tip where the pedal furrows converge, and a more or less distinct triangular flap of tissue ("horn") over the caudal pit (Ziegltrum, 2000).

Nominal *Hemphillia* supposedly consists of two species groups. The first group comprises the relatively large taxa (length of adults greater than 4 cm) *Hemphillia camelus*, *H. danielsi*, *H. dromedarius*, and *H. malonei*. The second group consists of the relatively small taxa (length of adults 2.5 cm or less) *H. burringtoni*, *H. glandulosa*, and the dubious *H. pantherina*. Besides size differences between the groups, there are morphological differences of the tails (see Ziegltrum, 2000).

The two small jumping slug taxa *Hemphillia burringtoni* and *H. glandulosa*, both native to the area covered by the Northwest Forest Plan, are of particular ecological interest as they represent an important dispersal mechanism for fungal species, which are crucial for forest health.

Hemphillia burringtoni was originally described by Pilsbry (1948) as a new subspecies of *H. glandulosa* and later elevated to full species status based on anatomical differences (Branson, 1972). *H. burringtoni* has been observed in copulation with *H. glandulosa* and many variations of "intermediates" have been found (Ziegltrum, 2000). The uncertain species status has important consequences for management recommendations, as these recommendations require the involved species to be identifiable. However, this has proven to be very difficult as recent field observations (see Ziegltrum, 2000) raise the question, whether the anatomical differences proposed by Branson (1972) are valid (e.g. *H. burringtoni* lacks distinct papillae on the mantle and has a smooth penial stimulator whereas *H. glandulosa* has distinct papillae and a wrinkled penial stimulator).

The current uncertain status of the two *Hemphillia* taxa led to the following statement by Ziegltrum (2000): "It is important to note that there are taxonomic questions concerning these two organisms. Uncertainty exists in field identification characteristics between the two species, as compared to literature descriptions. Numerous intermediate specimens have been found. The question concerning whether *H. glandulosa* and *H. burringtoni* are two distinct species needs to be answered."

Given these taxonomic and systematic uncertainties, we here use two maternally inherited mitochondrial DNA makers (COI and 16S), one bi-parentally inherited nuclear DNA marker (ITS1) as well as morphological (external) and anatomical (internal) characters to:

1) infer the phylogenetic position of *Hemphillia glandulosa* and *H. burringtoni* within the genus *Hemphillia* as well as within the family Arionidae,
2) study the phylogenetic relationships between populations of the two nominal taxa *Hemphillia glandulosa* and *H. burringtoni*,
3) study population dynamics relative to potential genetic breaks, phylogeographic patterns and intra-population differentiation,
4) study the degree of morphological and anatomical differentiation within and between taxa,
5) test the usefulness of morphological and anatomical markers previously used to distinguish between the two species, and
6) evaluate the systematic and taxonomic status of nominal *H. glandulosa* and *H. burringtoni*.

2. Materials and methods

2.1. Specimens studied

The study includes a total of 199 specimens of *H. glandulosa* Bland & Binney 1872 and *H. burringtoni* Pilsbry 1948 from 24 sites throughout their distribution areas (Fig. 1, USDA Forest Service, 2003). All of them were used for morphological studies, 118 (59 for each of the nominal taxa) for molecular studies and of those, 84 for anatomical studies.

In addition, 45 other representatives of the family Arionidae were used for phylogenetic studies (bringing the total number of individuals studied genetically to 163). They include four other nominal species of the genus *Hemphillia* (*Hemphillia malonei* Pilsbry 1917, *Hemphillia camelus* Pilsbry & Vanatta 1897, *Hemphillia danielsi* Vanatta 1914, and *Hemphillia dromedarius* Branson, 1972) as well as representatives of the genera *Prophysaon, Zacoleus, Gliabates, Ariolimax, Udosarx*, and *Arion*. Four other taxa studied here could not be assigned to an existing group and likely represent new genera. They are labeled as genus A, B, C, and D.

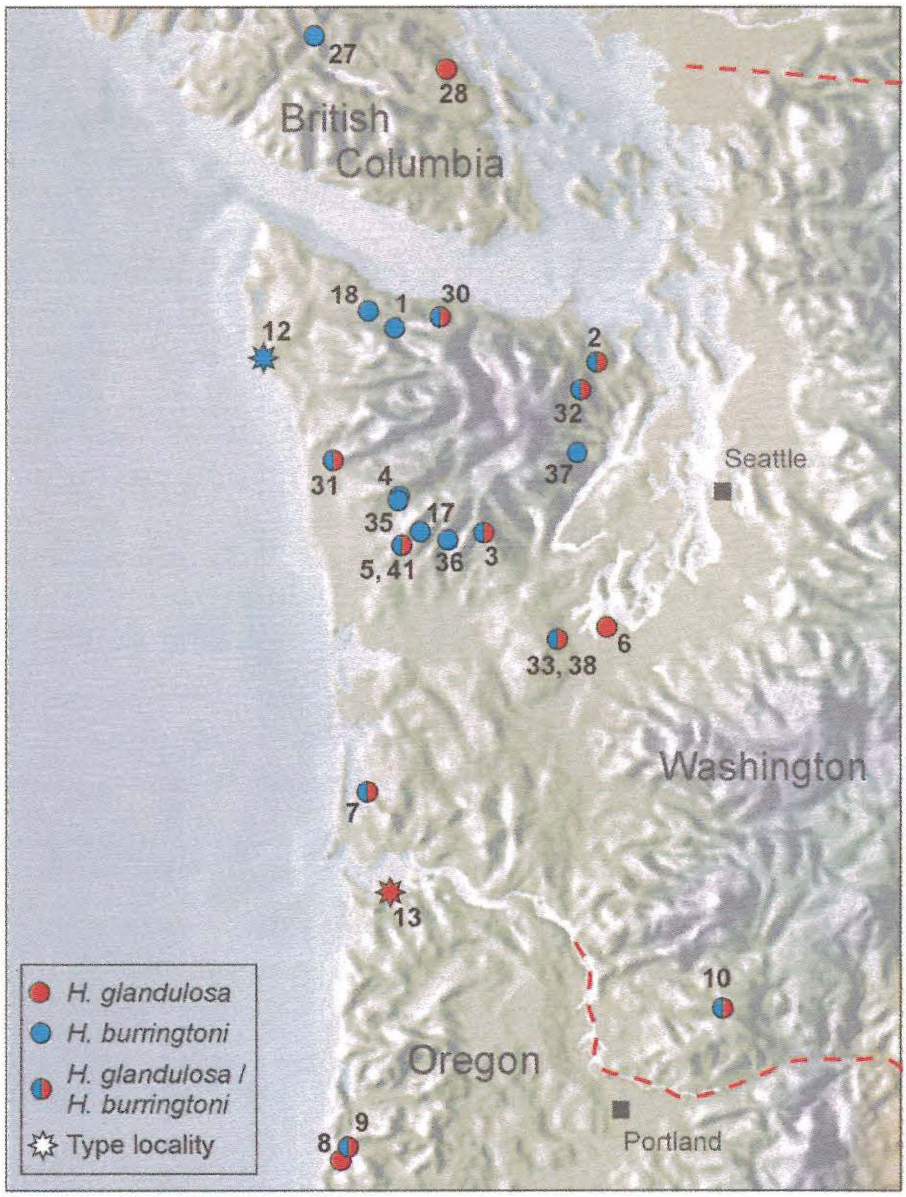

Fig. 1: Collecting sites for *Hemphillia glandulosa* and *H. burringtoni*. For detailed locality information and key to locality numbers see USDA Forest Service, 2003.

Species assignment of *H. glandulosa* and *H. burringtoni*

Initial species identification of the live animals was based on the presence or absence of a row of dark, gray dots above the pedal furrow. Specimens exhibiting one or more of these dots were identified as *Hemphillia burringtoni,* while those lacking dots above the pedal furrow were assigned to *Hemphillia glandulosa*. This approach has been used by the USDA Forest Service and most field biologists before, as this one morphological characteristic was the most distinctive in differentiating between the two nominal species.

Pilsbry (1948) described *Hemphillia glandulosa burringtoni* as a new subspecies, differing from *H. glandulosa* by its smooth mantle and gray dots in the pedal groove. *H. burringtoni* was elevated to species status by Branson (1972), based on differences in genitalia, lack of papillosity of the mantle and a row of 17–23 black spots immediately above the pedal furrow.

Since we examined live specimens, we relied solely on the morphological characteristics, and found that the degree of papillosity of the mantle was not a helpful characteristic in 199 specimens we collected. Only five of these had a smooth mantle, and one of the specimens died within a few hours after it was observed. Two of the five smooth mantled slugs had dots above the pedal furrow; the remaining three did not have any dots. Therefore, in order to differentiate the specimens, we used the presence or absence of dark, gray dots above the pedal furrow for species identification.

Processing of specimens

Individual specimens were placed in a small, air-free container filled with water at room temperature. Then the container was transferred into a refrigerator and remained there for 2–3 hours until the specimens were relaxed (but still alive). After that the specimens were transferred in a cold solution of 80% ethanol with a slug:ethanol ratio of at least 1:10. The solution was changed after a few hours for the larger specimens or after one or two days for the smaller once. The specimens were kept in the refrigerator until processing.

2.2. Molecular analyses

DNA isolation, PCR, and sequencing

The method described in Spolsky et al. (1996) was used for isolating DNA from individual slugs. The primers to amplify a fragment of the COI gene with a target length of 655 base pairs (excluding 51 bp primer sequence) are LCO1490 and HCO2198 as described by Folmer et al. (1994). The primers for amplification of a 16S rRNA fragment with a target length of 411-420 bp (excluding 42 bp primer sequence) are 16Sar-L and 16Sbr-H of Palumbi et al. (1991). For amplification of a 459-475 bp long ITS1 fragment (excluding 42 bp primer sequence), the primers of Armbruster et al. (2000) were utilized.

Sequences (forward and reverse) were determined using the LI-COR (Lincoln, NE) DNA sequencer Long ReadIR 4200 and the Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ).

The protein-coding COI sequences were aligned unambiguously by eye using BioEdit 5.0.9 (Hall 1999). The initial alignments of the 16S and ITS1 sequences using default settings in ClustalX (version 1.81; Thompson et al. 1997) were refined manually.

Sequences were aligned to produce three different datasets: A) for various representatives of the family Arionidae (16S, COI; dataset 1), B) for specimens of *Hemphillia glandulosa* and *H. burringtoni* (16S, COI; dataset 2), and C) for specimens of *Hemphillia glandulosa* and *H. burringtoni* (ITS1; dataset 3) (see Appendix I). The 16S portion of the Arionidae dataset (dataset

1) had 8 highly variable regions that roughly corresponded to alignment-ambiguous loop regions in the secondary structure model for the dorid slug *Thecacera pennigera* (Thollesson 1999). It was not possible to unambiguously align these regions even when using secondary structure information. As the accuracy of phylogenetic inference crucially depends on the accuracy of sequence alignment, we excluded this region from subsequent analyses.

Preliminary statistical analyses

Several studies have shown that the highly variable COI gene is prone to transitional saturation at or above the family level (e.g. Wilke et al., 2001). We therefore used the test of Xia et al. (2003), as implemented in the software package DAMBE (Xia & Xie, 2001), to test whether our Arionidae dataset (dataset 1) showed a significant level of saturation. The test revealed a substantial degree of saturation. In order to mitigate the effect of saturations, 3rd codon positions were excluded from the COI data portion (see Appendix I). A Xia et al. (2003) test using the reduced COI dataset did not reveal a significant degree of saturation.

To examine whether there are significant differences in incongruence length between the COI and 16S portions in datasets 1 and 2, the HOMPART command in PAUP* v. 4.10b (Swofford, 2002) was used to perform homogeneity-partition tests (Farris et al., 1995). As the tests did not reveal significant differences, the 16S and COI data were used in a combined analysis.

A permutation tail probability test (PTP) (Archie, 1989) was used to measure phylogenetic signals in our three dataset. The PTP test showed in all cases a significant cladistic covariance, indicating that the cladograms could not have arisen by chance alone. Based on these results, our three datasets were considered to be suitable for further analyses.

Nucleotide diversities (differences within groups of sequences) and divergences (differences among groups of sequences), both corrected according to the K2P-parameter-model, were calculated using MEGA 2 (Kumar et al., 2000) with standard errors estimated by 1,000 bootstrap replications with pairwise deletion of gaps and missing data.

Phylogenetic reconstruction

Prior to the phylogenetic analysis, the computer program Modeltest 3.06 (Posada & Crandall, 1998) was used in order to find the optimal model of DNA substitution. The models selected are listed in Appendix I.

For phylogenetic reconstruction, we used the software package MrBayes 3.0b4 (Huelsenbeck & Ronquist, 2001). The program is based on Bayesian inference, an approach that performs exceptionally well in supporting correct grouping compared to traditional non-parametric bootstrap methods (Alfaro et al., 2003).

First, we compared several independent runs for each dataset using the default random tree option and the starting parameters suggested by Modeltest to monitor the convergence of the ln likelihoods of the trees. Based on these preliminary analyses, we did final runs using the Metropolis-coupled Markov chain Monte Carlo variant with four chains (one cold, three heated) and 1,000,000 sampled generations with the current tree saved at intervals of 10 generations. Finally, a 50% majority rule tree was constructed from all sampled trees with the first 500 to 2,000 trees (= 5,000 to 20,000 generations) ignored as burn in (see Appendix I).

Spatial analysis of molecular variance (SAMOVA)

In order to investigate the spatial population structure of *H. glandulosa* and *H. burringtoni* specimens and to find the location of important genetic barriers, we used the program SAMOVA

1.0 (Dupanloup et al., 2002), which implements an approach to define groups of populations that are "geographically homogeneous and maximally differentiated from each other genetically". SAMOVA was chosen because, unlike classical tests of genetic structure, it finds a group structure based solely on genetic data and does not require an a priori definition of groups based on non-genetic traits (Dupanloup et al., 2002).

2.3. Morphological and anatomical analyses

Morphological work

The morphological work was conducted on 199 specimens. Characters studied were: body length, mantle length, width of shell, papillosity of mantle, markings on mantle, parallel lines on foot, reticulation on foot, dots above pedal furrow, caudal horn, keel as well as color values and chroma of head, optic tentacles, sole, foot, and mantle using standardized Munsell Soil Color Charts.

Anatomical work

Dissections were done using a Wild M-5 dissecting microscope equipped with an ocular micrometer. 84 specimens were dissected in the current study; detailed anatomical drawings were prepared from 18 specimens.

The animals were pinned in a water bath with the ventral surface down and external body and shell proportions were studied. Then mantel and shell were removed to expose the mantel cavity. For a detailed study of the reproductive system and other internal organs, the dorsal body well was carefully cut open with a median, dorsal, longitudinal incision from the anterior end of the mantel cavity to the snout. Later the digestive gland was removed to expose the ovotestis. During the dissection, we tried to avoid changing the position of internal organs so that the measurements and drawings reflect the actual topology of the specimens studied as much as possible.

Principal components analysis (PCA)

Principal components analyses (PCA) were performed from "quantified" morphological variables of *H. glandulosa* and *H. burringtoni* to construct a new set of orthogonal coordinate axes such that the projection of points onto them have maximum variance. The input data matrix is given in Appendix II. The analyses were done with the software package NTSYSpc 2.02g (Applied Biostatistics Inc.). First we standardized the input data, as the results of a PCA are sensitive to the units of measurement in a study. Then we computed the correlations among the standardized variables and generated a similarity-dissimilarity matrix from which principal components were extracted until the eigenvalue became < 1. Finally, the objects were projected onto various combinations of PC axes.

Discriminant analysis (DA)

The standardized anatomical variables were used to perform discriminant analyses (DA), with STATISTICA 5.1 (StatSoft Inc.). The mtDNA lineage-type of the respective population resulting from genetic analysis (lineages are indicated by Roman numerals in Fig. 3) was applied as a grouping variable in DA. The analysis was done in order to identify those morphological characters that would support the major groups found in the genetic analyses.

3. Results

3.1. Molecular analyses

Molecular phylogeny of arionid taxa

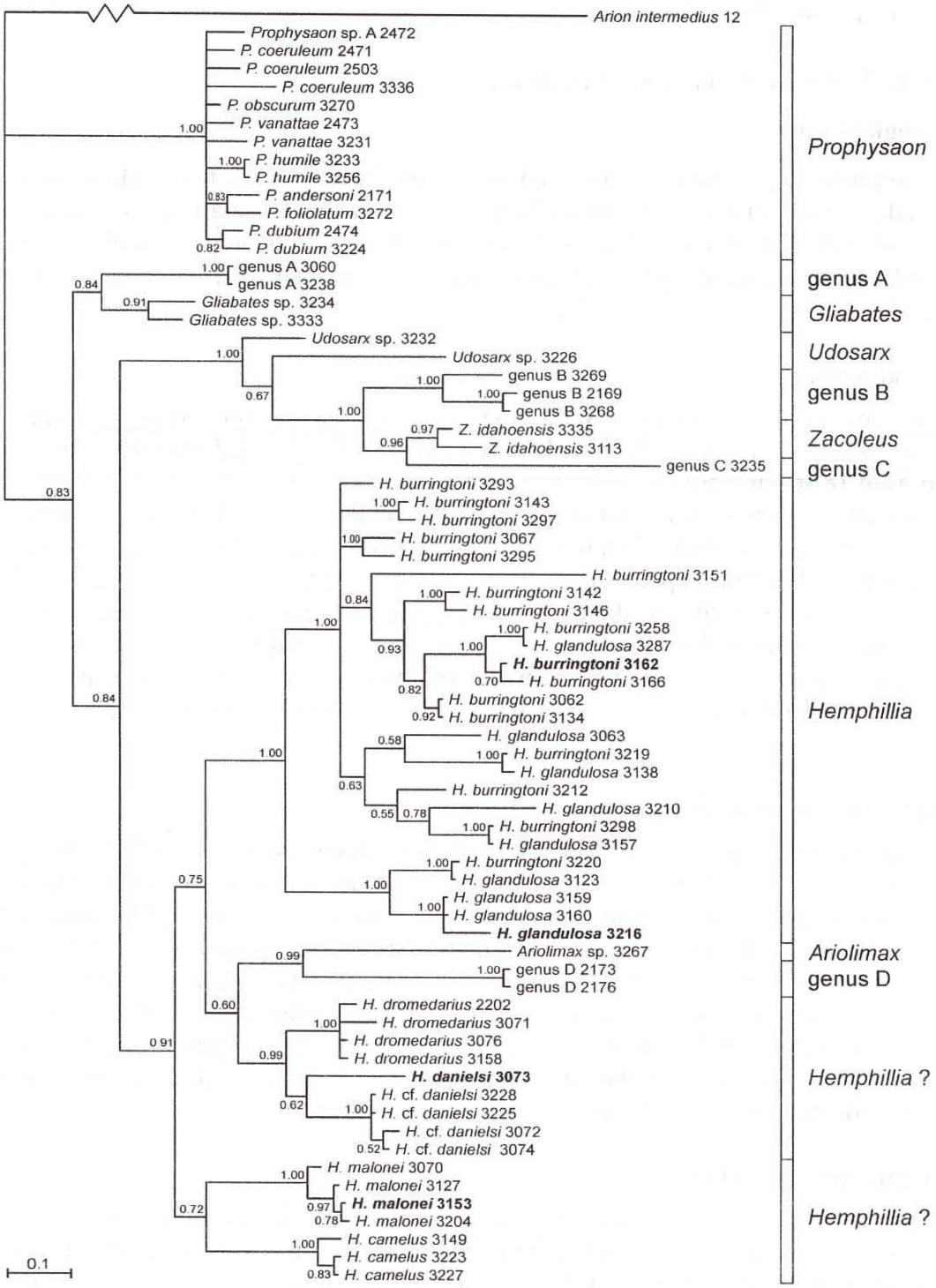

Fig. 2: Bayesian phylogram for arionid taxa based on combined fragments of COI DNA and 16S rRNA showing the 50% majority-rule consensus of topologies sampled during the Bayesian search. The scale bar represents the substitutions per site according to the model of sequence evolution applied. Posterior probabilities are provided for each clade. Topotypes are shown in bold. Genus-level groups are indicated by white bars.

A phylogenetic tree for 71 individuals of the family Arionidae based on the two mitochondrial genes for COI and 16S is presented in Fig. 2 (for parameters of phylogenetic reconstruction see Appendix I). As mentioned above, 8 highly variable regions in the 16S portion of the dataset were removed, as they were difficult to align. We also removed 3rd codon positions from the COI data portion due to problems with saturation. Because of the associated decreased degree of heterogeneity, the resulting phylogeny may not well reflect relationships within or between closely related taxa, but might be optimized for higher-level relationships, i.e. on the genus level.

The phylogeny comprises four major ingroup-clades and twelve sub-clades (Figure 2). These sub-clades appear to represent genus-level groups with four of them (labeled genus A–D) potentially being new. The first major clade is formed by representatives of the genus *Prophysaon*. The second clade comprises the genus *Gliabates* as well as a new genus from northern Idaho here called 'genus A' (a formal description of this new genus was submitted to the journal Zootaxa by Leonard et al.). The third clade consists of the genera *Udosarx* and *Zacoleus* as well as a *Zacoleus*-like group from Washington and Oregon (genus B) and a potentially new genus from Queen Charlotte Islands, British Columbia (genus C). Genetically the most diverse clade is the fourth clade, comprising representatives of the genera *Hemphillia* and *Ariolimax* as well as a potentially new genus (genus D) from north-central California that morphologically resembles representatives of the genus *Prophysaon* (Wilke & Davis, 2000). Within this fourth clade, taxa of the genus *Hemphillia* do not form a monophyletic group. Instead, taxa of *Ariolimax* and genus D cluster together with *Hemphillia danielsi* and *H. dromedarius* whereas representatives of the two nominal species *H. glandulosa* and *H. burringtoni* as well as representatives of *H. malonei and H. camelus* each form distinct clades (Fig. 2). Acknowledging the relatively low support values for some of the nodes (e.g. only 75% for the split of the *H. glandulosa/H. burringtoni* complex and the clade comprising *Ariolimax*, genus D and *H. danielsi/H. dromedarius*), this is the strongest evidence yet that nominal *Hemphillia* may consist of more than one genus.

Phylogenetic relationships of *Hemphillia glandulosa* and *H. burringtoni*

Mitochondrial DNA (COI, 16S)

Among the 118 specimens of *H. glandulosa/H. burringtoni* studied, a total of 63 haplotypes was found for the combined COI/16S fragments. The average nucleotide diversity (corrected to the K2P-model) among all individuals of *H. glandulosa/H. burringtoni* is 0.1622±0.0082 (note that this is a very high value that typically would reflect species- or even genus-level relationships in some other groups of mollusks). The average divergence between specimens from different sites ranges from 0.0003±0.00035 (between sites 35 and 37) to 0.2434±0.0173 (between sites 17 and 18).

A phylogram of the combined COI/16S data set reflecting the 50% majority-rule consensus of topologies sampled during the Bayesian search is shown in Fig. 3. The ingroup taxa of *H. glandulosa/H. burringtoni* form a monophyletic group with a posterior probability of 1.00. Populations of *H. glandulosa/H. burringtoni* cluster in several distinct clades and lineages. There are two major clades (I and II; see Fig. 3) and fifteen sub-clades and lineages with pairwise K2P-distances of > 0.05 (numbered A–P). The two major clades I and II are characterized by a nucleotide divergence of 0.1981±0.0111; the nucleotide diversity for clades I and II are 0.1486±0.0081 and 0.0902±0.0055, respectively. The spatial distributions of the major clades and the sub-clades are indicated in Fig. 4. Clade I reaches from central Oregon along the Pacific Coast into the Olympic Peninsula and Vancouver Island. Clade II is restricted to Northern Oregon and central Washington. Acknowledging the limited number of sites studied, the two major clades do not appear to be overlapping.

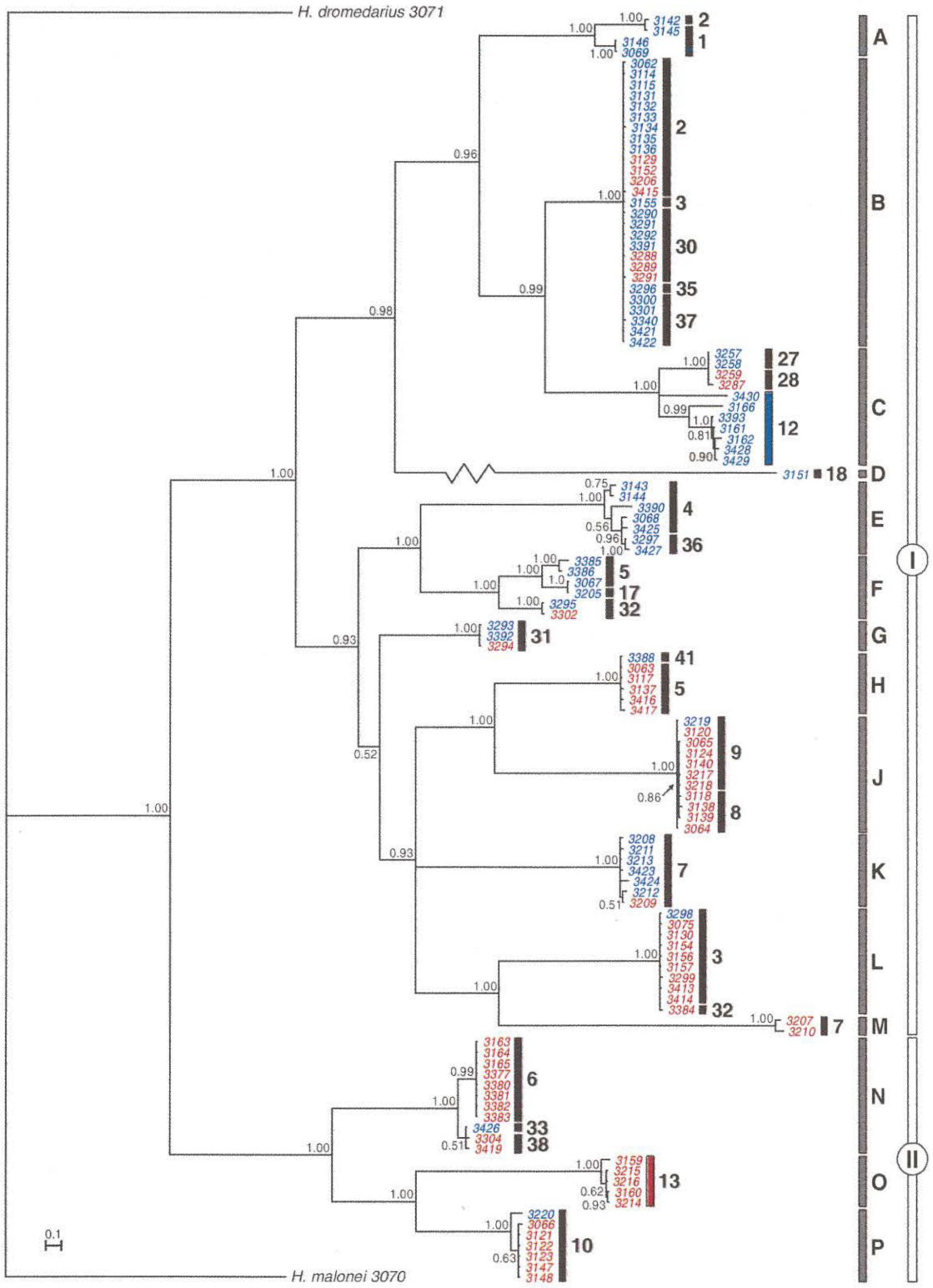

Fig. 3: Bayesian phylogram for individuals of nominal *Hemphillia glandulosa* (red DNA preparation numbers) and *H. burringtoni* (blue DNA preparation numbers) based on combined fragments of COI DNA and 16S rRNA showing the 50% majority-rule consensus of topologies sampled during the Bayesian search. The scale bar represents the substitutions per site according to the model of sequence evolution applied. Posterior probabilities are provided for each clade. Collecting sites are encoded with bold Arab numbers (topotypes of *H. glandulosa* and *H. burringtoni* are indicated by red and blue bars, respectively). The two major ingroup-clades are indicated by Roman numbers; sub-clades by letters. For a key to locality and DNA preparation numbers see USDA Forest Service (2003).

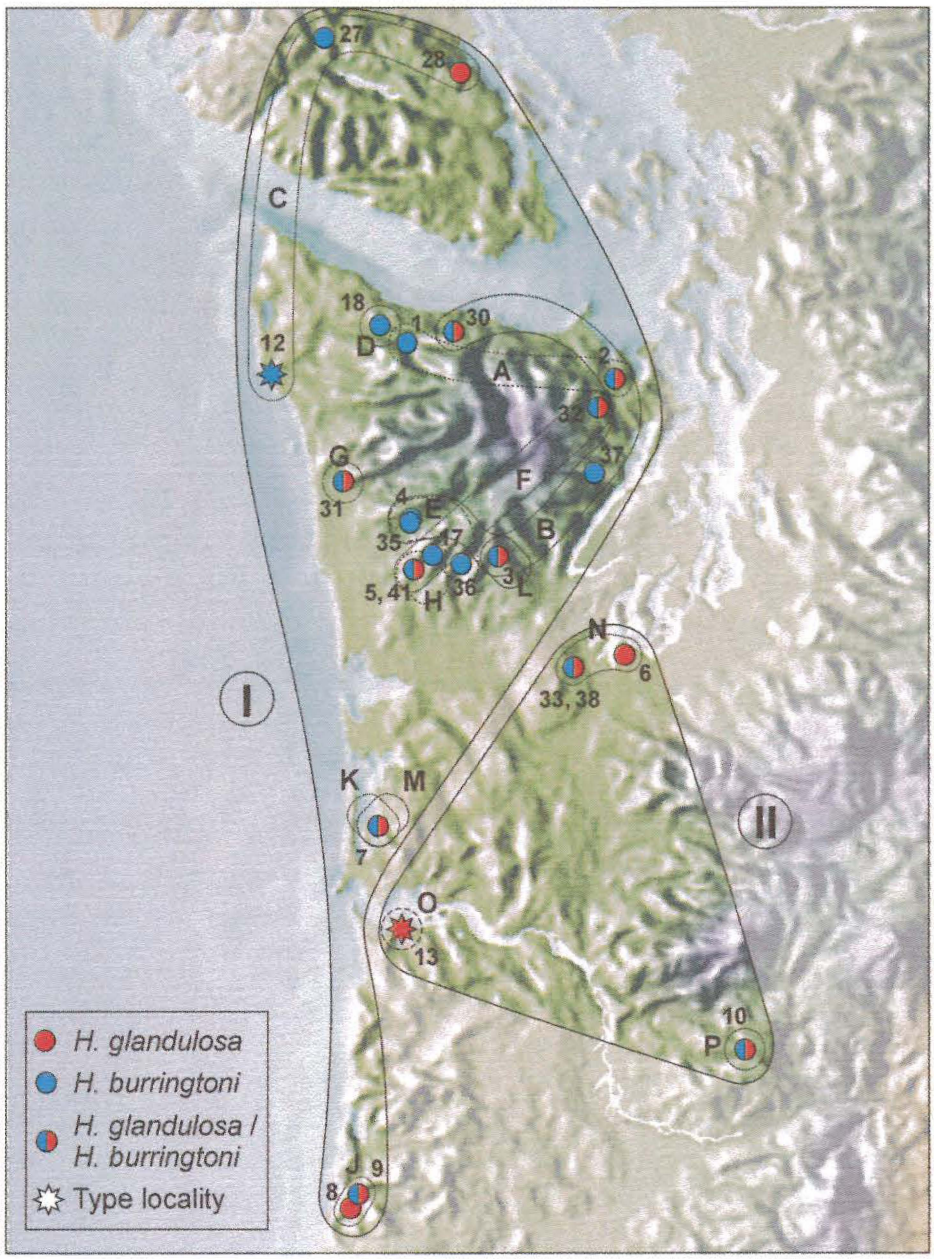

Fig. 4: Geographic distribution of major genetic clades and sub-clades of *Hemphillia glandulosa* and *H. burringtoni* from Fig. 3. Collecting sites are encoded with Arab numbers. The two major clades are indicated by Roman numbers; sub-clades by letters. For a key to locality numbers see USDA Forest Service (2003).

Eight of the fifteen sub-clades are geographically restricted to single sites and three more clades are restricted to adjacent sites. The only relatively widely distributed sub-clades are clades A, B, C, and F (see Fig. 4).

At four of the 24 sites studied, we found individuals belonging to more than one genetic sub-clade. Individuals from site 2 either belonged to clade A or B; individuals from site 3 to clades B or L; individuals from site 5 to clades F or H; and individuals from site 7 to clades K or M.

Neither the genetic tree in Fig. 3 nor the distribution map of genetic clades (Fig. 4) indicates a correspondence between the (morphologically determined) individuals of *H. glandulosa* and *H. burringtoni* and genetic cluster patterns. In fact, often individuals of the two nominal species share the same haplotype (see Fig. 3). However, it has to be noted that all specimens from the type locality of nominal *H. glandulosa* (site 13 in Fig. 3) belong to major clade II, whereas all specimens from the type locality of nominal *H. burringtoni* (site 12 in Fig. 3) belong to major clade I (also see Fig. 4).

Nuclear DNA (ITS1)

The average nucleotide diversity (corrected to the K2P-model) among all individuals of *H. glandulosa/H. burringtoni* is 0.0459±0.0055. A Bayesian phylogram of the ITS1 data set is shown in Fig. 5. There are four sub-clades (a–d) and two major clades (I, II). The two major clades are characterized by a nucleotide divergence of 0.0779±0.0105; the nucleotide diversity within clades I and II are 0.0342±0.0049 and 0.0268±0.0063, respectively.

These two major clades in our ITS1-phylogeny are in complete concordance with the two major clades in the mtDNA phylogeny (Fig. 3). Also, some of the sub-clades show a fair degree of concordance. Sub-clade a in the ITS1-phylogeny corresponds to sub-clades A–D in the mtDNA phylogeny, sub-clade c (ITS1) to sub-clades J+L (mtDNA), and sub-clade d (ITS1) to sub-clades N–P (mtDNA). However, a relatively low degree of concordance was found between specimens clustering in clade b (ITS1) and the corresponding specimens in the mtDNA phylogeny. In fact, in the mtDNA phylogeny three of the specimens (3211, 3207, and 3210) that belong to clade b in the ITS1-phylogeny are intermixed with specimens that belong to clade c in the ITS1-phylogeny. Acknowledging that the number of specimens studied for the ITS1-phylogeny is too low for explicit tests of recombination, there is some evidence for hybridization among specimens from relatively closely related clades. For example, specimens 3211 and 3210 (both from site 7) belong to two different (but closely related) clades in the mtDNA phylogeny (see Fig. 3). In the ITS1-phylogeny, they cluster together, possibly indicating recombination. However, not all specimens from sites with more than one distinct mtDNA clade show signs of recombination. Specimens 3063 and 3067 from site 5 that belong to clades H and F in the mtDNA phylogeny also cluster distinctly apart in the ITS1-phylogeny.

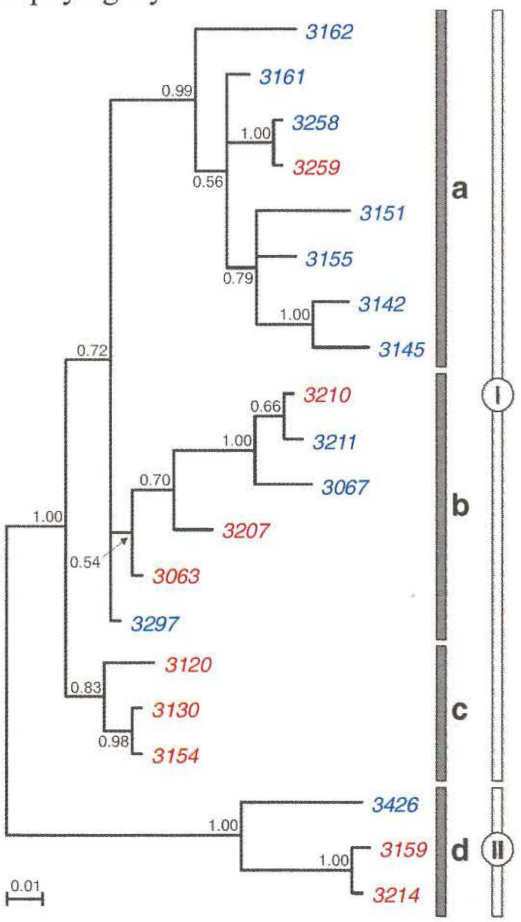

Fig. 5: Bayesian phylogram for individuals of nominal *Hemphillia glandulosa* (red DNA preparation numbers) and *H. burringtoni* (blue DNA preparation numbers) based on a fragment of the nuclear ITS1 gene. The scale bar represents the substitutions per site according to the model of sequence evolution applied. Posterior probabilities are provided for each clade. Two major clades are indicated by Roman numbers; sub-clades by letters. For a key to DNA preparation numbers see USDA Forest Service (2003).

Spatial analysis of molecular variance (SAMOVA)

The strong geographic subdivision we have found between clades I and II in the Bayesian analysis (see Figs. 3, 4) was confirmed using SAMOVA, a tree-independent approach. SAMOVA indicated a geographic subdivision and significant genetic barrier ($P < 0.001$) between individuals from sites 6, 10, 13, 33, and 38 (corresponding to clade II in Fig. 4) and all other sites studied here (corresponding to clade I in Fig. 4).

3.2. Morphological and anatomical analyses

Principal components analysis (PCA)

The first six axis of the PCA on all standardized variables accounted for 17.0%, 13.6%, 10.9%, 9.2%, 7.4% and 6.4% of total anatomical variation, respectively. They all had eigenvalues ≥ 1. Plots of various combinations of principal components did not show distinct morph-spaces corresponding to *Hemphillia glandulosa* and *H. burringtoni*. Examples involving PC's 1 and 2 as well as PC's 2 and 3 are shown in Fig. 6.

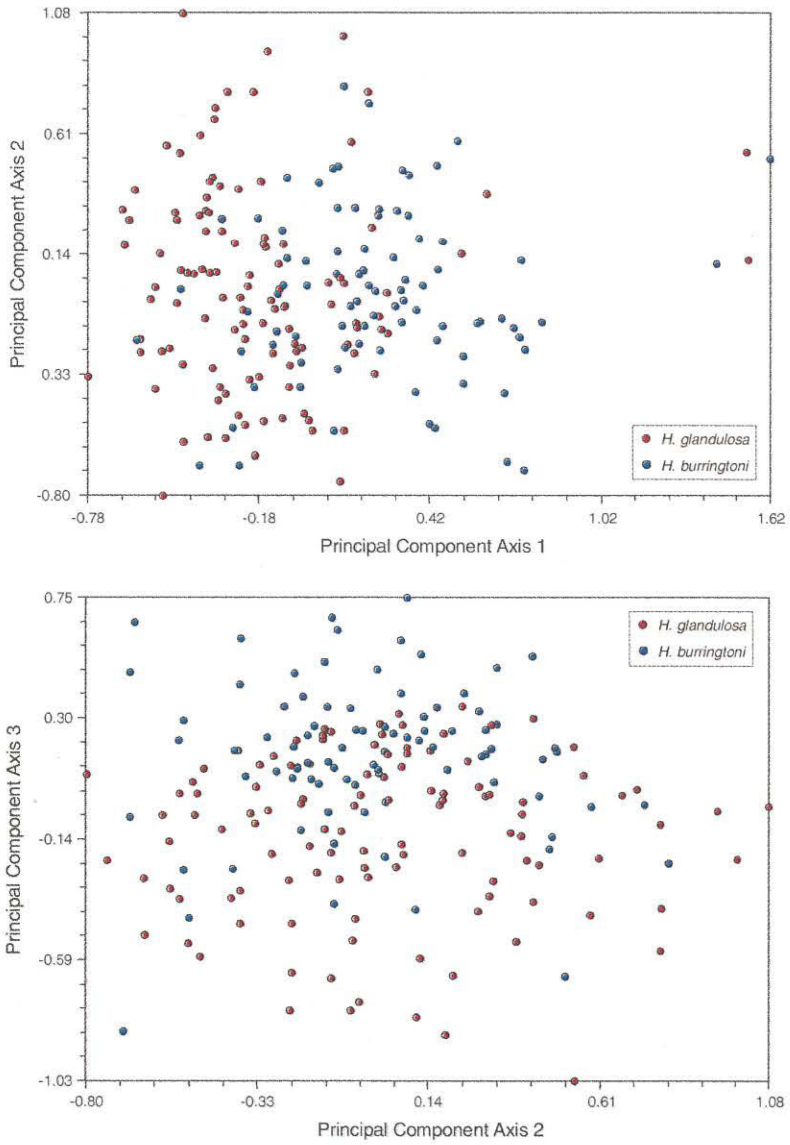

Fig. 6: Plots of principal components 1 and 2 (top) and 2 and 3 (bottom) on all morphological variables for *Hemphillia glandulosa* and *H. burringtoni*.

Discriminant analysis (DA)

As the PCA did not show distinct morph-spaces corresponding to *Hemphillia glandulosa* and *H. burringtoni* based on the morphological characters that were originally used to distinguish between the two taxa, we used discriminant analysis to find those morphological characters that support the genetic grouping of the specimens studied here into clade I (which contains topotypes of *H. burringtoni*) and clade II (containing topotypes of *H. glandulosa*).

The grouping after the two major mtDNA lineages (see Fig. 2) did not result in a significant discrimination model (P = 0.125, Wilk's Lambda = 0.618), indicating that the morphological characters studied here did not contribute to a discrimination model that corresponds to the major DNA groups. Fig. 7 shows individuals from DNA clades I (white circles) and II (black circles) and their actual classification based on morphological characters. From 90 individual of clade I, 85 were correctly assigned to this group by the discriminant analysis and 5 individual were wrongly assigned to group II (upper row in Fig. 7). From the 22 individuals of clade II, 13 were correctly and 9 wrongly assigned (lower row in Fig. 7).

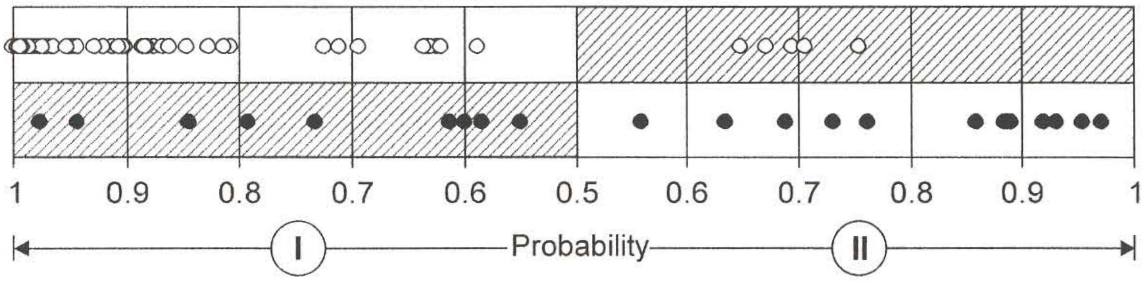

Fig. 7: Classification of individuals of DNA clades I (white circles) and II (black circles) based on a discriminant analysis of morphological characters. Incorrect assignments are indicates by hatchings. The discrimination model is not significant.

Anatomical differentiation

The detailed anatomical studies of 84 specimens of *Hemphillia glandulosa/H. burringtoni* did not reveal significant anatomical differences among specimens from different sites, among specimens from different genetic clades or among specimens that were assigned to either species based on morphological characters. All specimens studied had a very similar anatomical groundplan. Pilsbry (1948) suggested that *H. glandulosa/H. burringtoni* differ in that *H. glandulosa* has a smooth penial stimulator, whereas its surface is corrugated in *H. glandulosa*. Acknowledging that we studied the penial stimulator in only few specimens, one of the topotypes of *H. burringtoni* dissected did have a penial stimulator with a rather corrugated surface, possibly indicating that this character might not be useful for distinguishing among the two nominal species.

Anatomical drawings of topotypes of *H. glandulosa* and *H. burringtoni* are shown in Fig. 8. Though there are no qualitative anatomical differences, there is a fair degree of quantitative anatomical variation relative to form and size of internal organs. This is particularly evident in the reproductive system (e.g. penis, epiphallus, spermatheca, spermoviduct, and hermaphroditic duct). However, these differences are not restricted to the inter-population level, as similar differences can be observed within population. To demonstrate the degree of intra-population variation, we show drawings of the spermatheca of two topotypes of *H. glandulosa* (DNA preparation numbers 3159 and 3160) in Fig. 9. Note that these specimens are not only from the same population and were collected at the same time, they are also genetically almost identical (see Fig. 3).

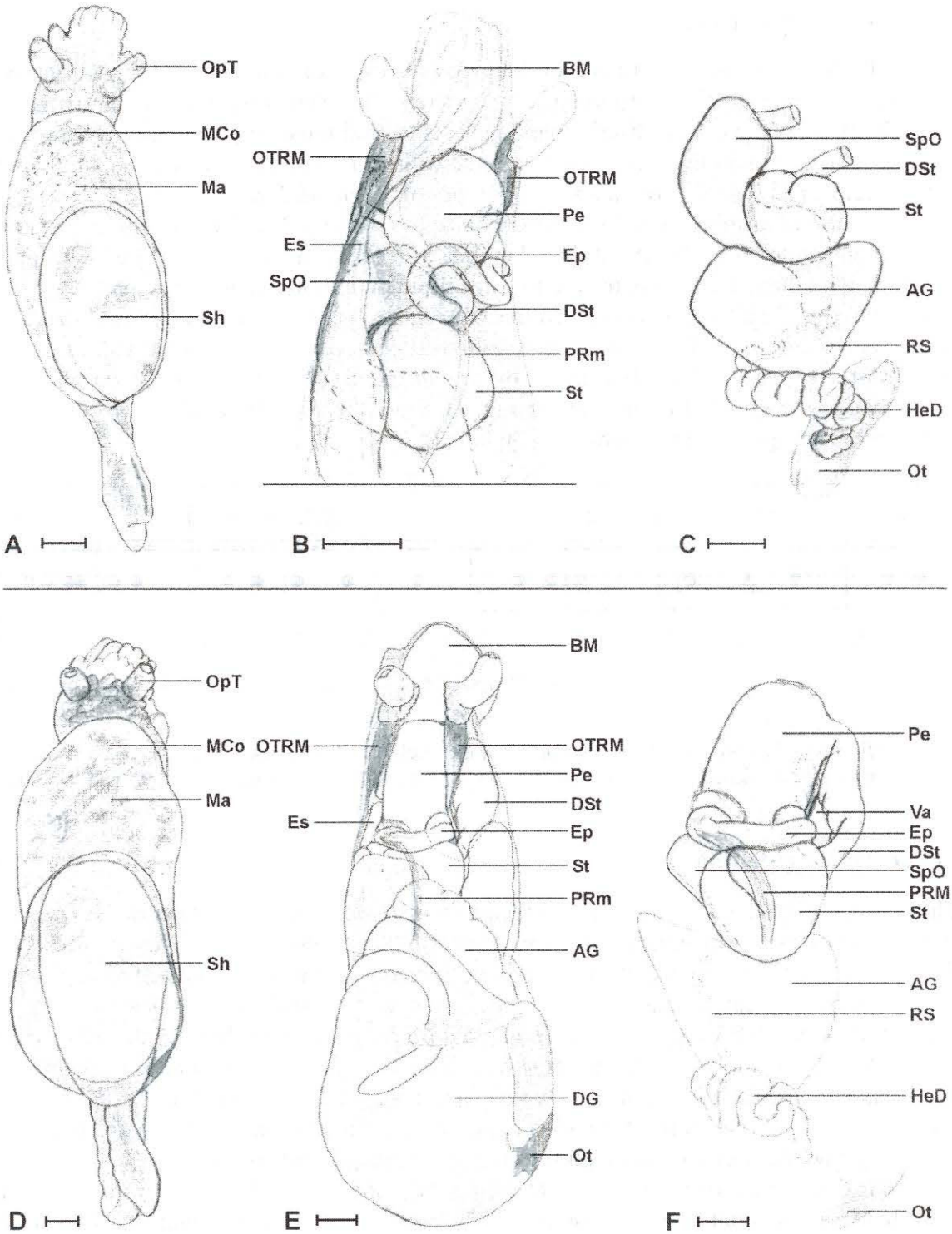

Fig. 8: Anatomical drawings of topotypes of *Hemphillia glandulosa* (A–C; DNA preparation 3159) and *H. burringtoni* (D–F; DNA preparation 3166). A, D: dorsal view of ethanol preserved specimens; B, E: dorsal view of dissected specimens with mantle removed; C, F: dorsal view of reproductive system. Note that the digestive and albumen glands were removed in B (but not in E) and penis and vagina were removed in C (but not in F). The scale bars represent 1 mm (AG=albumen gland, BM=buccal mass, DG=digestive gland, DSt=duct of spermatheca, Ep=epiphallus, Es=esophagus, HeD=hermaphroditic duct, Ma=mantel, Mco=mantel collar, OpT=optical tentacle, Ot=ovotestis, OTRM=optical tentacle retractor muscle, Pe=penis, PRm=penis retractor muscle, RS=receptaculum seminis, Sh=shell, SpO=spermoviduct, St=spermatheca, Va=vagina).

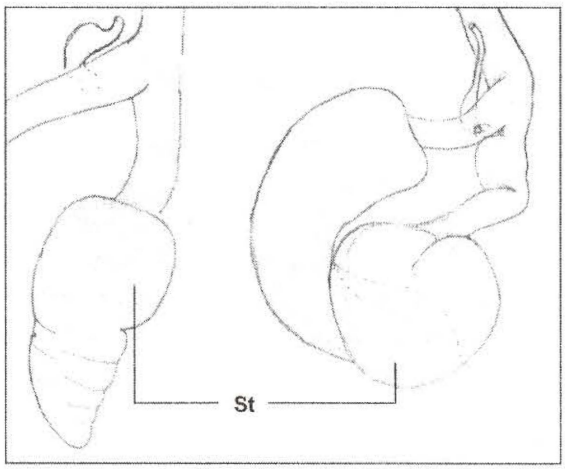

Fig. 9: Size and shape variations of the spermatheca (St) in specimens from the same site (left *H. glandulosa* topotype 3160, right *H. glandulosa* topotype 3159).

Specimens from sites 1, 4, 10, and 36 showed strong signs of degeneration of the reproductive system, e.g. spermatheca empty and elongated, albumen gland greatly reduced in size, vagina and spermoviduct very slim, and hermaphroditic duct very thin, de-coiled and discolored. In some specimens, the female side of the reproductive system showed a stronger degeneration than the male side. A specimen of *Hemphillia burringtoni* where both male and female sides show a strong degree of degeneration is shown in Fig. 10.

All specimens with the degenerated reproductive system were collected in July, a time when the reproductive season has ended and when most specimens die off. Note that all other specimens studied here were collected during the reproductive season from November to January.

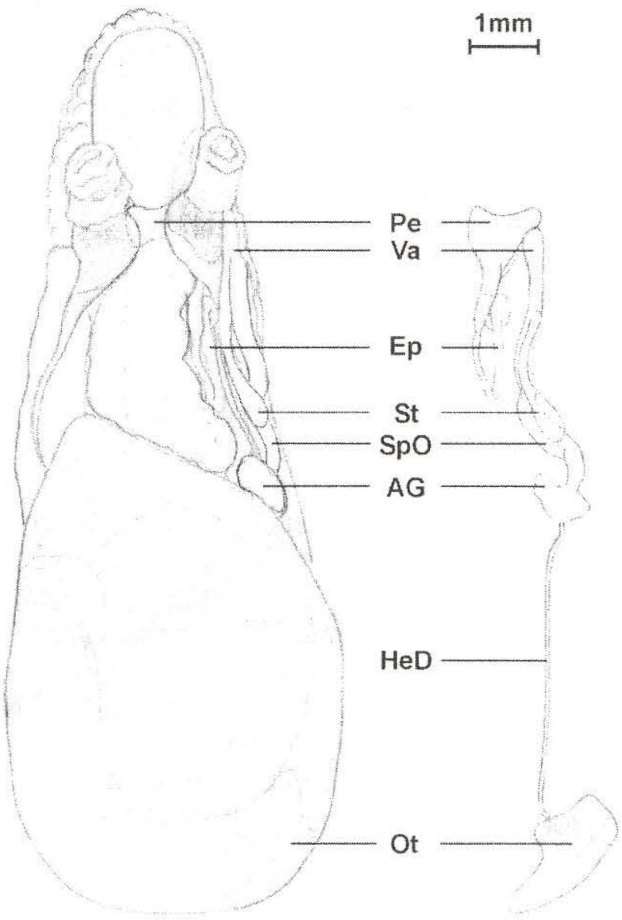

Fig. 10: Anatomical drawings of a *Hemphillia burringtoni* specimen (DNA preparation number 3146) with degenerated reproductive system. Left: dorsal view of dissected specimen with mantle removed, right: dorsal view of reproductive system (AG=albumen gland, Ep=epiphallus, HeD=hermaphroditic duct, Ot=ovotestis, Pe=penis, SpO=spermoviduct, St=spermatheca, Va=vagina).

4. Discussion

4.1. Molecular phylogeny of the genus *Hemphillia*

Traditionally, the genus *Hemphillia* has been considered to consist of two species groups: the relatively small taxa *H. burringtoni*, *H. glandulosa*, and the dubious *H. pantherina* as well as the relatively large taxa *Hemphillia camelus*, *H. danielsi*, *H. dromedarius*, and *H. malonei*. Our molecular study confirms the distinctness of the *H. burringtoni/H. glandulosa* complex. We were unable to study *H. pantherina* as this taxon is only known from its original description and was never found again. However, we also discovered major genetic differences within the large *Hemphillia* taxa, where *H. malonei* and *H. camelus* cluster distinctly apart from *H. danielsi* and *H. dromedarius*. Moreover, representatives of other genera (*Ariolimax* and a new, *Prophysaon*-like genus from California) cluster within *Hemphillia* and render this group polyphyletic. Acknowledging the relatively low support values for some of the clades in Fig. 2, we hypothesize that nominal *Hemphillia* consists of three genera: A) *H. burringtoni* and *H. glandulosa*, B) *H. dromedarius* and *H. danielsi*, and C) *H. malonei* and *H. camelus*. As *H. glandulosa* is the type species of the genus *Hemphillia* Bland & Binney 1872, both *H. glandulosa* and *H. burringtoni* would remain in that group. However, new genus names would have to be found for nominal *H. dromedarius* and *H. danielsi* as well as for nominal *H. malonei* and *H. camelus*. It should be noted that the classification of *Hemphillia* into three distinct genetic groups is supported by preliminary anatomical analyses (Thomas Burke, pers. comm.).

4.2. Molecular genetics of *Hemphillia glandulosa* and *H. burringtoni*

The 118 specimens of *Hemphillia glandulosa* and *H. burringtoni* cluster in two major groups in the phylogenetic analysis of mtDNA. These groups do not reflect the previous morphology-based assignment of these two taxa. Instead, there is a relatively strong correlation between these two clades and their geographic distribution (a finding that is corroborated by SAMOVA). Clade I is restricted to the northern and western part of the known range of *H. glandulosa* and *H. burringtoni*, whereas clade II is restricted to the central-eastern part (Fig. 4). Note that the type locality of *H. burringtoni* is within the geographic area of clade I, whereas the type locality of *H. glandulosa* is within the area covered by clade II. Within these two major clades, there is a total of fifteen sub-clades and lineages, all with pairwise K2P-distances of > 0.05 (see Fig. 3). Though genetic distances should not be used alone to make taxonomic decisions and there is no universal threshold that would indicate species-level relationships, we found in previous studies that K2P-distances of > 0.02 (> 2%) often reflect species-level relationships (e.g. Wilke et al., 2000). Thus, there is a strong possibility that at least some of these groups (labeled A–P in Fig. 3) represent distinct species. Also, it should be noted that the distances among many of these groups are as great or greater than distances among other "good" species currently assigned to *Hemphillia* (e.g. *H. dromedarius/H. danielsi*). However, as some of these sub-clades are geographically not well separated (with sub-clades occurring in sympatry at some sites) and as mtDNA data alone might be misleading when studying species-level relationships (e.g. Ballard et al. 2002), we also studied the nuclear ITS1 gene in selected specimens. Overall, the ITS1-phylogeny shows the same high degree of heterogeneity that was found in the mtDNA data (see Fig. 5). Moreover, the two major mtDNA clades are well reflected in the nuclear phylogeny as well as many of the sub-clades. There is no sign of recombination among distantly related clades. However, recombination is evident in some, but not all, of the closely related clades. Though recombination is a hotly

debated topic in evolutionary biology and systematics, under many species concepts, this would possibly indicate that some of the sub-clades may not represent distinct species.

Nevertheless, the incredible genetic diversity found in the current study gives important insights into the evolution of the *H. glandulosa* and *H. burringtoni* species complexes. Many groups found are strongly localized and the mtDNA distance among geographically closely related groups is in part as high as 0.20 or 20% (a value that would reflect genus-level relationships in many other gastropod groups; e.g. Wilke, 2003).

Preliminary molecular-clock analyses (details not shown here) indicate that, even when applying an extremely fast mtDNA clock rate, all the fifteen sub-clades and lineages are at least 2 million years old. Moreover, given the relatively low degree of intermixing of specimens from different genetic clades, the respective population very likely remained at the same place for a long period of time and probably survived the whole Pleistocene period in mountain refugia (e.g. ice-free valleys of the Olympic Peninsula). Pleistocene survival of invertebrate taxa has been described from the Pacific Northwest before (mainly in refugia along the Pacific coast). But the potential large-scale survival of slug species in mountainous areas, as indicated by our study, is a very significant finding that might help to shed new light on the evolutionary biology of invertebrate species in the Pacific Northwest. It also should be noted that the actual genetic diversity within *H. glandulosa* and *H. burringtoni* might be even higher than reported here, as studies of additional specimens from new sites likely will reveal more genetic lineages.

4.3. Morphological and anatomical differentiation of *Hemphillia glandulosa* and *H. burringtoni*

Our morphological and anatomical studies are conclusive. The morphological characters previously used to distinguish between *H. glandulosa* and *H. burringtoni* (notably the papillosity of the mantle and black dots above the pedal furrow) do not allow for a reasonable differentiation between the two species (see the results of the PCA in Fig. 6). Moreover, even a discriminant analysis, which uses a *post hoc* prediction to separate among groups and always allows for a better discrimination than *a priori* methods, does not result in a significant discrimination model. In other words, even with knowing the genetic grouping of the specimens involved, we were not able to identify those morphological characters that would support such a grouping. This does not necessarily mean that morphological characters, in general, are useless for species differentiation; it only means that the characters studied here and the methods applied might not be appropriate for distinguishing between *H. glandulosa* and *H. burringtoni*. However, given the relatively large number of specimens studied and the paucity of morphological characters in arionid slug, it appears to be very unlikely that future studies will reveal robust morphological characters that could be used for field-identification. We therefore recommend refraining from using morphological characters for species assignment of *H. glandulosa* and *H. burringtoni*.

This recommendation might also apply to anatomical characters. We were unable to find robust differences among specimens from the two major clades, yet among specimens from the genetically distinct sub-clades. Though there is a fair amount of quantitative differences in our datasets, there is also a relatively high amount of anatomical variation within population (see Fig. 9), possibly obscuring inter-population differences. This is not a problem specific to slug species, but has been observed in many other cryptic gastropod groups before (e.g. Wilke et al., 2002).

In addition to the problem of intra-population variations, there is a considerable degree of anatomical change over the lifetime of the slugs studied here. Specimens from most other gastropod groups remain reproductively active until they die and once the reproductive system is fully developed, anatomical changes are relatively minor. However, specimens of *Hemphillia glandulosa/H. burringtoni* show signs of strong degeneration of the reproductive system after the

end of the reproductive season, making it even more difficult to find robust anatomical characters for species-level studies.

4.4. Systematic and taxonomic status of *Hemphillia glandulosa* and *H. burringtoni*

All previous descriptions for *Hemphillia glandulosa* and *H. burringtoni* are relatively vague as to the morphological/anatomical differentiation and the potential geographic separation of the taxa. As mentioned above, we were not able to confirm the proposed morphological/anatomical differences. Moreover, Pilsbry (1948) reported a wide range for *H. glandulosa* (from northern Oregon to British Columbia) but only a single site for *H. burringtoni*; another finding that is refuted by our study. However, as noted above, all topotypes of nominal *H. glandulosa* (site 13 in Fig. 3) belong to major clade II, whereas all topotypes of nominal *H. burringtoni* (site 12 in Fig. 3) belong to major clade I (also see Fig. 4).

4.5.Conclusions

A) Topotypical *Hemphillia glandulosa* is part of major genetic clade II and topotypical *H. burringtoni* is part of clade I. These two clades are therefore defined by those taxa.

B) There are potentially several other undescribed species in each of these major clades, the exact number of which remains unknown. Therefore, we recommend referring to taxa in clade I as the "*Hemphillia burringtoni* species-complex" and to taxa in clade II as the "*Hemphillia glandulosa* species-complex".

C) The genus *Hemphillia* is not monophyletic. Some taxa previously assigned to *Hemphillia* like *H. dromedarius* and *H. danielsi* as well as *H. malonei* and *H. camelus* likely belong to different (new) genera. Genuine *Hemphillia* appears to comprise only the *Hemphillia burringtoni* and *H. glandulosa* species complexes studied here.

D) There are no known morphological or anatomical characters that could be used to distinguish between taxa from the two species complexes. Thus, we suggest differentiating between the two species groups solely on the basis of geographic information (i.e. taxa within the geographic range of clade I should be assigned to the *Hemphillia burringtoni* species-complex and taxa within the range of clade II to the *Hemphillia glandulosa* species-complex, see Fig. 4). Given that there are potential geographic contact points between clade I and II, we suggest a small follow-up study to determine the exact geographic boundary between the two clades.

E) Pleistocene survival has strongly affected evolutionary processes in both the *Hemphillia burringtoni* and *Hemphillia glandulosa* species-complexes. Despite the unknown systematic status of most of the species involved, the radiations observed here are of great value for a general understanding of speciation processes in the Pacific Northwest and the strongly localized subclade-lineages deserve full protection.

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6. Literature cited

Alfaro, M.E.; Zoller, S. & Lutzoni, F. (2003): Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov chain monte carlo sampling and bootstrapping in assessing phylogenetic confidence. Molecular Biology and Evolution 20: 255–266.

Archie, J.W. (1989): A randomization test for phylogenetic information in systematic data. Systematic Zoology 38: 239–252.

Armbruster, G.F.J.; van Moorsel, C.H.M. & Gittenberger, E. (2000): Conserved sequence patterns in the non-coding ribosomal ITS-1 of distantly related snail taxa. Journal of Molluscan Studies 66: 570–573.

Ballard, J.W.O.; Chernoff, B. & James, A.C. (2002): Divergence of mitochondrial DNA is not corroborated by nuclear DNA, morphology, or behavior in *Drosophila simulans*. Evolution 56: 527–545.

Branson, B.A. (1972): *Hemphillia dromedarius*, a new arionid slug for Washington. Nautilus 85: 100–106.

Dupanloup, I.; Schneider, S. & Excoffier, L. (2002): A simulated annealing approach to define the genetic structure of populations. Molecular Ecology 11: 2571–2581.

Farris, J.S.; Källersjö, M.; Kluge, A.G. & Bult, C. (1995): Testing significance of incongruence. Cladistics 10: 315–319.

Folmer, O.; Black, M.; Hoeh, W.; Lutz, R. & Vrijenhoek, R. (1994): DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299.

Hall, T.A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Huelsenbeck, J.P. & Ronquist, F. (2001): MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17: 754–755.

Kumar, S.; Koichiro, K.; Jakobsen, I. & Nei, M. (2000): MEGA: Molecular evolutionary genetics analysis, version 2.0. Pennsylvania State University, University Park, and Arizona State University, Tempe.

Palumbi, S.; Martin, A.; Romano, S.; Mcmillian, W.O.; Stice, L. & Grabowski, G. (1991): The simple fool's guide to PCR. University of Hawaii, Honolulu.

Pilsbry, H.A. (1948): Land Mollusca of North America (north of Mexico), vol. 2(2). The Academy of Natural Sciences of Philadelphia Monograph 3: 521–1113.

Posada, D. & Crandall, K.A. (1998): Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818.

Spolsky, C.; Davis, G.M. & Yi, Z. (1996): Sequencing Methodology and Phylogenetic Analysis: Cytochrome b Gene Sequence reveals Significant Diversity in Chinese Populations of Oncomelania (Gastropoda: Pomatiopsidae). Malacologia 38: 213–221.

Swofford, D.L. (2002): PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates Inc., Sunderland.

Thollesson, M. (1999): Phylogenetic analysis of dorid nudibranchs (Gastropoda: Doridacea) using the mitochondrial 16s rRNA gene. Journal of Molluscan Studies 65: 335–353.

Thompson, J.D.; Gibson, T.J.; Plewniak, F.; Jeanmougin, F. & Higgins, D.G. (1997): The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876–4882.

Wilke, T. (2003): *Salenthydrobia* n.gen. (Rissooidea: Hydrobiidae): a potential relict of the Messinian salinity crisis. Zoological Journal of the Linnean Society 137: 319–336.

Wilke, T. & Davis, G.M. (2000): DNA analysis of mollusk species in Oregon: molecular phylogeny, taxonomy, and population structure of *Prophysaon coeruleum* (Gastropoda: Arionidae). Report to National Fish and Wildlife Foundation Project # 99-299.

Wilke, T.; Davis, G.M.; Falniowski, A.; Giusti, F.; Bodon, M. & Szarowska, M. (2001): Molecular systematics of Hydrobiidae (Gastropoda: Rissooidea): testing monophyly and phylogenetic relationships. Proceedings of the Academy of Natural Sciences of Philadelphia 151: 1–21.

Wilke, T.; Pfenninger, M. & Davis, G.M. (2002): Anatomical variation in cryptic mudsnail species: statistical discrimination and evolutionary significance. Proceedings of the Academy of Natural Sciences of Philadelphia 152: 45–66.

Wilke, T.; Rolán, E. & Davis, G.M. (2000): The mudsnail genus *Hydrobia* s.s. in the northern Atlantic and western Mediterranean: a phylogenetic hypothesis. Marine Biology 137: 827–833.

USDA Forest Service (2003): Hemphillia database. Olympic National Forest, Olympia, Washington.

Xia, X. & Xie, Z. (2001): DAMBE: Software Package for Data Analysis in Molecular Biology and Evolution. The Journal of Heredity 92: 371–373.

Xia, X.; Xie, Z.; Salemi, M.; Chen, L. & Wang, Y. (2003) An index of substitution saturation and its application. Molecular Phylogenetics and Evolution 26: 1–7.

Ziegltrum, J. (2000): Draft Management recommendations for two species of the genus *Hemphillia*. V. 2.8. Olympic National Forest, Olympia.

Appendix I

Information on the molecular datasets used in the present study and parameters for phylogenetic reconstruction.

	Dataset 1	Dataset 2	Dataset 3
Taxa	Arionidae	*H. glandulosa*, *H. burringtoni*	*H. glandulosa*, *H. burringtoni*
Gene fragments	16S + COI	16S + COI	ITS1
Total number of individuals	71	120	20
Highly variable regions excluded	yes (16S)	no	no
3rd codon positions excluded	yes (COI)	no	no
Total length of aligned sequences	730 bp	1069 bp	502 bp
Model and parameters of sequence evolution used for Bayesian inference	GTR+I+G Base=(0.2485 0.1545 0.2189) Rmat=(1.7629 8.8012 5.9978 1.5991 18.0728) Rates=gamma Shape=0.6997 Pinvar=0.5993	GTR+I+G Base=(0.3317 0.0995 0.1327) Rmat=(1.0547 6.0940 1.5044 1.2042 11.1406) Rates=gamma Shape=0.5084 Pinvar=0.4438	SYM+G Base=equal Rmat=(0.9729 1.3004 1.5681 0.3568 2.1431) Rates=gamma Shape=0.0664
Sampled generations during Bayesian search	1,000,000	1,000,000	1,000,000
Number of trees ignored as burn-in	2000	1000	500
Corresponding figures	Fig. 2	Fig. 3	Fig. 5

Appendix II

Morphological measurements (columns 2-21) from 199 specimens of *Hemphillia glandulosa* and *H. burringtoni* used for the principal components analysis. For the discriminant analysis, only specimens with known assignment to genetic clades were used (last column). The data in columns 1-21 were kindly provided by J. Ziegltrum.

Specimen number	Body Length (mm)	Mantle Length (mm)	Width of Shell (mm)	Papillosity	Value of Head	Chroma of Head	Value of Optic Tentacles	Chroma of Optic Tentacles	Value of Sole	Chroma of Sole	Value of Foot	Chroma of Foot	Value of Mantle	Chroma of Mantle	Markings on Mantle	Parallel Lines on Foot	Reticulation on Foot	Dots Above Pedal Furrow L	Dots Above Pedal Furrow R	Keel	Genetic clade
004B	21	11	1	2	5	4	4	1	8	1	4	3	4	3	25	2	1	16	15	-	I
005B	23	11	2	1	5	3	5	1	7	1	4	3	4	2	30	2	1	12	17	-	I
006B	20	11	2	2	5	4	3	1	7	2	3	2	4	2	25	2	2	16	16	-	I
007B	23	10	2	2	5	6	5	1	7	1	5	2	5	3	25	1	3	17	16	-	I
010B	17	6	2	2	5	3	3	2	7	1	4	3	5	2	30	2	2	11	10	-	I
012B	20	11	2	2	4	3	3	2	7	1	4	2	5	3	20	2	2	14	17	-	I
014B	20	10	2	1	6	4	5	1	7	1	5	2	5	2	15	2	2	11	9	-	I
015B	15	8	1	1	5	3	4	1	7	1	4	2	5	3	20	1	3	13	13	-	
016B	22	11	1	1	5	4	4	1	7	2	4	3	5	2	20	2	2	15	15	-	I
017B	20	10	1	2	5	4	4	1	7	1	4	2	5	2	10	2	2	13	15	-	I
018G	21	11	2	2	5	3	6	1	8	2	6	4	6	1	7	1	1	0	0	-	I
019G	21	11	1	2	5	4	5	1	7	1	7	1	6	2	15	1	1	0	0	-	I
020G	18	10	2	2	6	3	7	1	7	1	6	3	6	1	15	2	2	0	0	-	
021G	17	9	1	2	6	4	6	1	7	1	5	2	5	1	20	1	3	0	0	-	I
025G	13	5	1	1	6	3	5	2	7	1	5	2	5	3	20	2	1	0	0	-	I
026G	13	7	2	1	6	4	4	1	7	2	5	3	4	1	20	2	1	0	0	-	
027G	15	7	2	2	6	4	6	1	8	1	8	2	5	1	20	1	2	0	0	-	I
028G	12	8	1	1	6	4	5	1	8	2	6	2	5	2	15	2	2	0	0	-	I
029G	14	7	2	1	6	3	5	1	7	1	5	2	5	3	10	1	2	0	0	-	I
030G	7	5	2	1	5	3	4	1	7	1	6	3	5	1	15	1	1	0	0	-	I
032G	12	6	2	1	6	2	4	1	7	1	3	1	3	2	25	2		0	0	-	
033G	12	7	2	2	6	4	5	2	7	1	5	2	5	2	25	2	2	0	0	-	I
034G	13	7	1	2	6	3	5	1	7	1	4	1	4	1	20	2	1	0	0	-	
035G	14	8	1	2	5	4	5	2	7	1	4	2	4	1	25	2	3	0	0	-	I
036B	20	10	1	1	6	4	4	1	7	1	5	1	4	1	30	2	2	2	4	-	II
037B	18	10	2	2	5	4	-	1	7	1	4	3	5	2	20	1	3	14	12	-	
038B	17	8	2	2	5	3	5	3	7	2	5	3	5	2	15	1	3	10	23	-	I
039B	23	11	1	2	6	4	5	1	7	1	5	4	5	2	10	1	3	6	8	-	I
041B	16	9	2	1	4	3	4	1	7	1	3	2	4	2	25	2	2	8	9	-	I
043B	-	10	1	2	4	3	5	2	8	2	3	2	4	1	25	2	2	19	18	-	I
044B	16	9	1	2	5	3	4	1	8	3	4	2	4	-	25	2	2	15	14	-	I
045B	17	9	2	1	6	4	5	1	7	2	3	1	4	3	25	1	3	13	15	-	I
046B	20	6	2	2	5	4	5	1	7	1	5	6	4	1	20	1	3	10	9	-	
048B	16	9	2	1	4	3	4	1	7	1	4	2	5	2	20	1	3	16	20	-	I
049B	15	8	1	2	4	3	4	2	7	2	5	4	6	1	20	2	2	19	19	-	I
050B	23	11	2	2	5	4	5	2	7	2	5	3	5	2	10	1	3	13	16	-	I
052G	15	8	2	1	6	4	5	1	7	1	4	2	5	2	15	1	3	0	0	-	II
053G	20	10	1	2	5	3	3	2	7	2	5	2	4	2	20	2	3	0	0	-	II
055G	19	10	2	2	6	3	3	2	7	2	4	1	3	1	30	2	2	0	0	-	II
056G	18	9	1	2	6	4	3	1	8	2	5	1	3	1	25	2		0	0	-	II

Specimen number	Body Length (mm)	Mantle Length (mm)	Width of Shell (mm)	Papillosity	Value of Head	Chroma of Head	Value of Optic Tentacles	Chroma of Optic Tentacles	Value of Sole	Chroma of Sole	Value of Foot	Chroma of Foot	Value of Mantle	Chroma of Mantle	Markings on Mantle	Parallel Lines on Foot	Reticulation on Foot	Dots Above Pedal Furrow L	Dots Above Pedal Furrow R	Keel	Genetic clade
057G	17	8	1	1	6	4	5	2	7	2	5	1	5	2	25	2	3	0	0	-	II
058G	11	8	2	1	6	4	5	1	7	1	4	2	4	2	10	2	3	0	0	-	I
088B	10	6	1	1	3	1	3	1	6	2	4	2	4	1	50	2	3	12	13	-	I
090B	19	11	2	2	5	2	4	1	7	2	5	1	4	1	40	2	2	15	16	-	
091G	12	7	2	1	5	2	5	1	7	1	5	2	5	1	60	2	2	0	0	-	I
092G	12	6	1	1	3	1	3	1	7	1	6	2	5	3	15	0	1	0	0	-	I
126B	25	13	2	2	5	1	4	1	7	2	4	3	5	3	25	2	3	22	20	-	
127B	25	12	2	2	5	1	4	1	7	2	5	2	4	1	40	2	2	24	23	-	I
130G	13	7	1	2	5	2	4	1	7	1	5	1	6	1	15	0	1	0	0	-	
131G	13	7	1	1	4	1	4	1	7	1	5	1	6	2	10	2	2	0	0	-	I
133B	20	12	2	2	6	1	4	1	7	1	5	1	5	2	20	2	2	10	15	-	
135B	25	9	1	2	5	1	6	1	7	2	5	1	5	1	30	1	3	12	14	-	
136B	17	9	2	2	6	1	4	1	7	1	5	1	5	2	20	1	1	8	11	-	
137G	13	7	2	2	5	1	6	1	7	2	4	1	4	2	30	2	2	0	0	-	I
140B	17	9	3	2	4	1	4	1	7	2	5	2	4	2	20	2	3	16	18	-	I
142G	26	12	2	2	5	3	5	1	7	3	4	1	5	2	25	2	2	0	0	-	I
143G	18	9	1	2	5	1	3	1	7	2	4	1	5	1	15	2	2	0	0	-	I
145G	23	11	2	2	5	2	3	1	7	4	4	2	4	3	25	1	2	0	0	-	I
146G	15	7	1	1	5	1	4	1	7	3	5	1	5	1	20	2	2	0	0	-	I
151G	17	7	2	2	6	1	5	1	7	1	6	1	6	1	15	2	1	0	0	-	I
152G	14	7	2	2	4	4	2	1	6	2	4	3	5	4	30	1	2	0	0	I	
157G	20	10	2	2	5	1	4	1	7	1	5	1	5	1	25	2	2	0	0	-	-
162B	17	8	2	2	6	3	5	1	7	2	5	2	5	2	15	2	2	4	6	-	I
163G	16	7	2	1	5	2	3	1	7	1	4	1	3	1	35	1	1	0	0	-	I
164B	13	7	3	1	3	1	3	1	6	2	4	2	3	2	30	1	1	9	5	-	I
165B	12	7	3	0	3	1	3	1	7	2	5	1	4	1	25	2	3	13	12	-	I
166G	18	10	2	2	4	3	3	1	7	2	5	1	4	1	20	2	3	0	0	-	I
167G	17	8	2	2	6	3	4	1	6	2	4	2	5	3	30	2	2	0	0	-	I
168B	17	9	2	2	6	3	6	1	7	2	6	3	6	2	7	1	1	8	12	-	I
169B	10	6	1	2	3	1	4	1	7	1	4	1	4	2	15	2	1	3	4	-	I
170B	18	8	2	2	5	2	5	1	7	2	5	1	4	1	20	2	2	12	13	-	I
171G	16	8	2	2	4	2	5	1	7	1	4	2	4	1	25	2	2	0	0	-	
172G	16	8	2	0	6	3	4	1	7	2	6	3	4	3	25	2	3	0	0	-	II
173G	17	9	3	1	2	1	3	2	6	2	3	2	5	4	30	2	2	0	0	-	II
174G	15	8	2	1	4	2	3	2	6	2	3	2	4	3	40	2	1	0	0	-	II
176G	18	8	2	2	4	1	3	1	7	2	5	2	4	2	30	2	3	0	0	-	II
177G	11	6	1	1	3	3	3	1	6	3	3	1	2	2	30	2	3	0	0	-	
178G	13	10	2	2	6	1	5	1	7	2	7	1	5	2	15	0	1	0	0	-	II
180G	12	7	2	1	4	4	2	2	6	3	3	3	3	2	25	2	1	0	0	-	
181G	16	8	2	1	5	1	3	1	7	2	4	2	4	2	20	2	3	0	0	-	
182G	15	10	2	2	5	3	4	1	7	1	6	1	5	1	20	2	3	0	0	-	
184G	16	8	2	2	6	3	6	1	6	2	4	3	4	3	20	0	1	0	0	-	
185G	14	7	2	2	5	3	5	2	6	2	4	3	3	3	25	0	1	0	0	-	
186G	16	8	1	2	3	2	4	1	6	2	3	2	4	2	25	2	1	0	0	-	
187G	-	15	8	1	5	2	5	1	7	2	5	2	4	2	25	1	3	0	0	-	
191G	12	7	1	2	6	3	5	2	6	3	5	3	4	3	20	0	1	0	0	-	
192G	12	7	2	2	5	4	3	2	6	2	4	3	4	4	25	1	1	0	0	-	

Specimen number	Body Length (mm)	Mantle Length (mm)	Width of Shell (mm)	Papillosity	Value of Head	Chroma of Head	Value of Optic Tentacles	Chroma of Optic Tentacles	Value of Sole	Chroma of Sole	Value of Foot	Chroma of Foot	Value of Mantle	Chroma of Mantle	Markings on Mantle	Parallel Lines on Foot	Reticulation on Foot	Dots Above Pedal Furrow L	Dots Above Pedal Furrow R	Keel	Genetic clade
193G	16	7	2	2	5	3	5	2	6	2	4	3	5	3	20	0	1	0	0	-	
195G	18	9	1	2	5	3	5	1	7	1	6	1	4	1	15	1	3	0	0	-	
197G	11	6	2	2	3	3	3	1	6	3	4	3	4	3	30	1	1	0	0	-	
198G	13	7	2	1	4	3	3	2	6	2	4	3	3	3	30	1	1	0	0	-	
200G	13	7	1	2	4	2	2	1	6	2	4	3	3	2	40	2	2	0	0	-	
202G	13	7	2	2	4	2	4	1	6	2	3	2	4	3	25	1	1	0	0	-	
204G	16	8	1	2	3	2	4	1	6	2	3	2	3	3	30	2	1	0	0	-	
208G	19	7	2	1	5	4	4	2	6	2	3	3	4	3	25	1	1	0	0	-	
212G	16	7	2	2	6	2	4	1	6	2	4	2	4	2	30	1	1	0	0	-	
215G	14	7	2	1	3	2	3	1	6	2	3	2	4	2	25	0	1	0	0	-	I
217G	10	6	2	2	5	2	3	1	6	2	4	2	4	2	20	0	1	0	0	-	
219G	15	8	1	2	5	1	4	1	7	1	5	1	4	1	25	0	1	0	0	-	
220G	13	7	2	2	4	3	3	1	7	2	3	2	4	3	30	2	2	0	0	-	I
221G	13	7	2	1	5	2	4	1	7	2	3	1	3	1	30	1	3	0	0	-	
222G	15	8	2	2	4	2	3	1	7	2	4	1	3	1	30	2	1	0	0	-	
223B	18	9	2	1	5	3	3	1	7	2	5	2	4	2	25	2	2	3	3	-	
224G	15	7	2	2	5	3	4	2	6	2	4	3	4	3	25	1	1	0	0	-	
225G	15	6	2	2	4	2	4	1	6	1	4	1	3	2	35	2	3	0	0	-	
226G	14	7	2	2	3	2	3	1	6	2	3	2	4	2	0	1	1	0	0	-	
227G	19	9	3	2	3	2	4	2	6	2	2	1	4	3	25	1	1	0	0	-	
231B	13	8	2	2	5	1	3	1	7	1	5	2	4	1	30	1	1	5	4	-	
232B	14	7	2	2	5	3	2	1	6	2	4	2	3	3	40	1	1	2	6	-	I
233B	20	9	3	2	5	3	3	1	7	3	3	2	4	3	30	2	3	18	17	-	II
235G	16	10	2	1	5	3	3	1	7	2	3	2	4	3	25	2	3	0	0	-	
236G	21	11	3	2	6	2	3	2	7	2	3	2	4	3	30	2	1	0	0	-	
237G	14	7	3	2	4	3	3	3	7	3	5	3	5	3	30	1	1	0	0	-	
238G	12	8	3	1	5	3	4	2	7	2	4	2	5	3	40	0	1	0	0	-	
239B	17	8	2	2	5	3	4	1	7	2	3	2	5	4	25	2	3	-	-	-	
240G	18	10	2	2	6	3	4	1	7	2	4	3	5	4	40	2	1	0	0	-	
241G	13	8	2	2	5	3	4	2	7	2	3	3	4	3	20	2	1	0	0	-	
242G	17	9	3	1	6	4	3	1	7	3	3	2	4	4	25	2	1	0	0	-	
243B	21	11	2	2	4	4	4	1	7	2	3	3	4	4	25	2	2	14	17	1	I
244B	16	9	3	1	5	4	3	1	6	2	3	3	5	4	30	1	1	12	14	0	
245B	17	12	2	2	5	3	4	2	7	2	4	2	5	3	25	1	3	13	19	1	I
246B	19	9	3	2	3	2	2	2	7	2	3	3	5	3	20	2	2	17	16	1	
247B	17	7	3	0	5	3	4	1	7	2	3	2	4	2	25	1	1	0	6	1	I
248B	25	10	3	1	5	3	4	2	7	2	4	2	5	3	30	1	1	15	16	1	
249B	22	10	2	2	5	3	3	2	7	2	3	2	5	3	20	1	2	5	0	1	I
250B	20	11	3	2	4	2	3	2	7	2	4	3	5	3	40	1	2	9	13	1	I
251G	19	10	3	1	5	3	4	2	7	3	3	3	4	3	30	2	2	0	0	1	
252G	16	8	2	1	5	3	4	1	7	2	3	3	4	3	25	2	2	0	0	1	
253G	15	8	2	0	5	3	4	1	7	2	3	2	4	3	40	2	2	0	0	1	
254B	19	10	3	2	3	2	3	1	7	2	3	2	4	4	40	2	2	13	15	1	I
255G	12	8	4	2	4	3	4	2	7	2	7	1	3	2	40	2	0	0	0	-	
256G	18	9	3	2	5	2	3	1	6	2	4	2	4	3	30	1	2	0	0	1	II
257G	19	9	2	1	5	3	3	1	6	2	3	2	4	2	50	2	2	0	0	1	II
258G	21	12	3	2	3	2	3	1	7	2	3	2	4	3	30	1	3	0	0	1	II

Specimen number	Body Length (mm)	Mantle Length (mm)	Width of Shell (mm)	Papillosity	Value of Head	Chroma of Head	Value of Optic Tentacles	Chroma of Optic Tentacles	Value of Sole	Chroma of Sole	Value of Foot	Chroma of Foot	Value of Mantle	Chroma of Mantle	Markings on Mantle	Parallel Lines on Foot	Reticulation on Foot	Dots Above Pedal Furrow L	Dots Above Pedal Furrow R	Keel	Genetic clade
259G	17	9	3	2	4	2	3	1	6	2	3	2	5	3	40	2	2	0	0	1	II
260G	23	14	3	2	4	2	4	1	6	2	4	1	4	2	25	2	3	0	0	1	II
262G	23	11	2	2	3	3	3	1	6	2	3	2	4	3	40	2	2	0	0	1	
263G	18	10	3	2	4	1	3	1	6	2	4	2	4	3	40	2	3	0	0	1	
264G	16	8	3	2	4	2	3	1	7	2	4	2	5	3	30	2	2	0	0	1	
265G	16	7	2	2	4	1	3	1	7	2	4	2	5	3	25	1	1	0	0	1	II
266G	18	10	2	2	3	1	3	1	6	2	4	2	4	3	30	2	2	0	0	1	II
267G	13	6	1	1	6	3	3	1	7	2	4	2	4	4	50	2	2	0	1	1	II
268B	21	10	3	2	4	4	3	2	7	2	3	2	5	4	25	2	2	22	17	1	I
289B	28	14	4	2	4	3	3	1	7	4	4	4	6	6	0	0	2	19	14	1	I
290B	25	14	4	2	5	6	5	2	7	6	5	6	5	4	0	2	2	17	19	1	I
291G	27	15	5	2	5	4	6	2	7	6	5	6	6	6	0	2	2	0	0	1	I
292G	28	13	5	2	4	4	4	2	7	6	4	4	5	4	0	1	1	16	20	1	I
293G	22	11	3	2	5	2	6	2	7	2	5	2	6	2	20	0	1	0	0	1	I
294G	22	10	3	2	6	2	7	2	7	2	5	2	7	3	20	0	1	0	0	1	I
295G	17	7	2	2	5	2	7	2	7	1	6	2	6	2	10	1	1	0	0	1	
296G	22	9	2	2	5	2	5	1	6	1	5	2	6	2	5	0	1	0	0	1	
297G	10	6	1	2	3	1	5	2	7	1	5	3	6	2	10	1	1	0	0	1	
299B	18	11	1	1	4	2	5	1	7	2	4	2	5	2	10	2	1	11	10	1	I
300G	19	9	2	1	6	2	4	1	7	2	4	1	5	2	15	2	3	0	0	1	
301B	18	9	2	1	3	2	3	1	7	2	4	1	3	2	20	2	2	10	11	1	
302G	19	9	3	2	4	2	3	1	7	3	4	2	5	3	30	2	2	0	0	1	I
303G	23	10	2	2	5	2	4	1	7	2	4	2	5	3	40	1	2	0	0	1	I
304B	15	7	2	1	4	2	3	1	7	3	3	2	4	3	40	2	2	11	11	1	
305B	22	9	4	1	4	1	4	1	7	3	3	2	5	3	40	2	2	4	8	1	I
306G	18	8	2	1	5	2	4	1	7	3	4	2	6	2	40	2	1	0	0	1	I
307B	21	8	3	1	6	3	4	1	7	3	4	2	5	3	40	1	1	8	16	1	I
308B	17	9	2	2	5	2	4	1	7	3	3	3	5	3	40	0	1	12	15	1	I
309B	18	9	1	2	5	2	3	1	6	2	4	2	5	3	40	2	2	6	9	1	I
310B	26	13	2	2	6	2	5	1	7	2	5	2	6	2	40	1	1	7	11	1	I
311G	26	12	4	2	6	3	5	1	7	3	5	2	7	3	40	0	1	0	0	1	
312B	14	8	2	2	5	2	3	1	7	1	4	2	5	2	40	1	1	8	7	1	
313B	22	11	3	1	4	2	3	1	7	2	4	2	5	2	40	0	1	21	16	1	I
314B	25	10	3	1	4	2	3	1	7	2	5	2	5	3	30	2	3	23	24	1	
315B	23	11	4	2	3	2	5	1	7	3	3	2	4	2	30	2	2	22	21	1	I
316B	23	12	2	1	4	2	6	1	7	2	4	1	5	3	40	1	1	13	12	1	I
317B	22	10	3	1	5	2	4	1	7	2	5	2	5	3	40	1	1	11	14	1	
318B	22	8	3	1	4	2	4	1	7	2	3	2	5	3	15	1	1	22	15	0	
319B	21	11	3	1	5	1	3	1	7	2	4	2	5	3	20	2	2	9	0	1	II
320B	26	11	3	2	4	2	3	1	7	2	3	1	5	2	20	2	3	20	18	1	I
321B	22	11	2	1	3	2	3	1	6	2	2	1	5	3	30	2	2	31	29	1	I
322G	22	10	2	1	5	1	4	1	7	2	4	1	5	2	25	2	2	0	0	1	
323B	33	13	3	1	3	2	4	1	7	2	3	2	5	2	25	2	2	15	10	1	I
324B	17	9	3	0	4	2	4	1	7	3	3	2	6	3	25	2	1	2	4	0	
325B	23	9	2	1	6	2	5	1	7	2	4	2	6	3	15	2	2	11	12	1	I
326B	22	9	2	1	4	2	3	1	7	2	3	2	5	2	25	1	1	8	9	1	
327B	17	7	2	1	5	2	2	1	7	2	4	1	5	2	30	1	1	5	5	1	I

Specimen number	Body Length (mm)	Mantle Length (mm)	Width of Shell (mm)	Papillosity	Value of Head	Chroma of Head	Value of Optic Tentacles	Chroma of Optic Tentacles	Value of Sole	Chroma of Sole	Value of Foot	Chroma of Foot	Value of Mantle	Chroma of Mantle	Markings on Mantle	Parallel Lines on Foot	Reticulation on Foot	Dots Above Pedal Furrow L	Dots Above Pedal Furrow R	Keel	Genetic clade
328G	12	7	2	2	4	2	3	1	7	2	5	2	6	2	20	1	1	0	0	1	I
329B	16	8	2	1	5	2	4	1	7	1	3	2	6	3	40	2	2	14	14	1	
330B	15	8	3	1	4	2	3	1	7	2	4	2	6	2	25	1	1	18	14	1	I
331B	21	8	2	1	3	1	3	1	7	2	4	2	5	2	30	1	1	12	13	1	
332B	16	8	3	1	5	2	4	1	7	2	4	2	6	2	40	-	-	12	16	1	I
333B	17	8	3	1	4	2	4	1	7	2	3	2	5	2	30	1	1	19	16	1	I
334B	22	9	3	1	5	2	3	1	7	2	4	2	6	2	30	2	1	12	14	1	I
335B	16	7	2	1	5	2	3	1	7	2	3	2	5	2	15	2	1	5	8	1	
336B	17	8	3	1	5	2	3	1	7	2	4	2	6	2	50	2	2	10	17	1	I
337G	16	7	2	2	5	2	3	1	7	2	5	1	6	3	40	2	2	0	0	1	
338G	23	11	3	1	5	2	4	1	7	2	4	2	5	2	30	2	2	0	0	1	I
339G	21	12	4	2	5	1	5	1	7	3	3	1	6	2	40	2	2	0	0	1	
340G	24	12	3	1	5	1	5	1	7	3	3	2	5	2	50	2	2	0	0	1	I
341G	13	7	2	1	4	1	4	1	7	1	4	1	6	2	25	1	1	0	0	1	
346G	8	5	1	1	4	1	3	1	7	2	3	1	4	1	20	0	1	0	0	1	
347G	14	7	1	1	5	1	4	1	7	2	5	3	5	2	30	1	1	0	0	1	II
348G	13	9	2	1	6	1	4	1	7	1	5	2	6	2	10	1	1	0	0	1	II
349B	20	10	2	2	5	1	3	1	7	2	4	1	5	2	25	1	3	0	3	1	